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Organization and Expression of Plasmodial Genes
Required for Erythrocyte Invasion

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Survival of the malaria parasite within its vertebrate is the result of multiple highly evolved mechanisms which allow the parasite to modulate or evade host defenses. Principle among these mechanisms is the ability to minimize contact between the parasite and the host immune system by remaining intracellular for the majority of its asexual life-cycle. For P. falciparum, invasion of host erythrocytes, as well as the sequestration of infected erythrocytes in the microvasculature, represent specialized mechanisms which are dependent upon receptor-ligand interactions between the parasite and host cells. As such these interactions represent models by which to address questions of cell-cell interactions at the molecular level and which represent rational sites for therapeutic intervention. The research focus of the laboratory is aimed at defining the biochemical and genetic basis for merozoite invasion of erythrocytes and sequestration of the infected erythrocyte. Studies are focussed on the organization and expression of P. falciparum genes involved in these mechanisms. Recent work is directed at chromosome structure and stability in P. falciparum,

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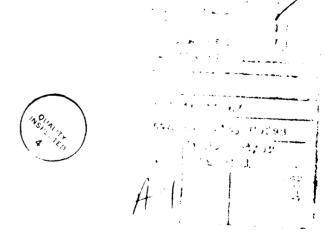with particular emphasis on the contribution of chromosomal rearrangement to novel mutational mechanisms in the parasite.

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Summary

Survival of the malaria parasite within its vertebrate is the result of multiple highly evolved mechanisms which allow the parasite to modulate or evade host defenses. Principle among these mechanisms is the ability to minimize contact between the parasite and the host immune system by remaining intracellular for the majority of its asexual life-cycle. For P. falciparum, invasion of host erythrocytes, as well as the sequestration of infected erythrocytes in the microvasculature, represent specialized mechanisms which are dependent upon receptor-ligand interactions between the parasite and host cells. As such these interactions represent models by which to address questions of cell-cell interactions at the molecular level and which represent rational sites for therapeutic intervention. The research focus of the laboratory is aimed at defining the biochemical and genetic basis for merozoite invasion of erythrocytes and sequestration of the infected erythrocyte. Studies are focussed on the organization and expression of P. falciparum genes involved in these mechanisms.

A gene for a glycophorin-binding protein of P. falciparum has been isolated and characterized and its role in the interactions of the merozoite with the human erythrocyte studied. Its potential as a protective immunogen against the erythrocytic stage of the parasite is under evaluation.

Sequestration of the P. falciparum infected erythrocyte is being studied by the characterization of the histidine rich knob protein, the gene for which has been cloned and sequenced. Analysis of knobless variants of the parasite revealed that chromosomal rearrangement can result in loss of expression of the gene. The contribution of chromosome rearrangement to mutational mechanisms in the parasite is under investigation.

Foreword

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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Body of Report

Four areas of research have been pursued in the last 12 months which summarize the specific goals of this program.

1) Role of the glycophorin-binding protein (130 kd) in erythrocyte attachment

2) Structure of the knob-associated histidine-rich protein (KAHRP) gene in knobby (K+) and knobless (K-) parasites

3) Regulation of expression of the KAHRP in K+ and K- parasites; role of chromosomal location on expression

4) Function of the KAHRP in knob formation and sequestration

Progress in these four areas will be summarized below along with work currently in progress to achieve these goals.

1) GLYCOPHORIN BINDING PROTEIN OF P. FALCIPARUM

The malarial parasite is dependent on the intracellular environment of its various host cells for growth and replication and is unable to survive extracellularly for any length of time. Thus invasion into host cells is a critical step in its life-cycle, the disruption of which can be used as a target for therapeutic attack. During its complex life cycle in its vertebrate host it invades two different host cells, initially the hepatocyte and subsequently the erythrocyte (1).

The erythrocyte is not a phagocytic cell, nor does it participate in endocytosis except in rare instances (2). Since cellular entry by the malarial parasite cannot be explained by conventional endocytotic pathways, it has been suggested that there are specific parasite proteins involved not only in recognition and binding to the erythrocyte surface, but also in deformation of the erythrocyte membrane leading to the formation of an endocytotic vacuole (3). A second feature of the parasite-erythrocyte interaction is the strict species specificity of invasion. There are over a 100 different species of the malarial parasite, plasmodium, and for the most part, merozoite invasion is restricted to the erythrocyte of the susceptible host animals. The specificity of the erythrocyte recognition has been taken to imply that entry is a receptor-mediated event. For Plasmodium falciparum, one of the four species of malaria that infects man, the erythrocyte receptors have been identified as glycophorin A and B, the major sialoglycoproteins of the erythrocyte surface (4,5,6,7). Genetic loss of glycophorin A from the erythrocyte (En(a⁻)cells), antibodies against glycophorin A or soluble glycophorin A (as well as specific tryptic fragments) all result in inhibition of merozoite invasion in vitro. Since other species of malarial parasites do not invade human erythrocytes or do not interact with glycophorin (8) it is likely that there are specific P. falciparum merozoite proteins that recognize human glycophorin. Two such glycophorin-binding proteins have been identified and localized to the merozoite surface. These proteins have molecular weights of 155,000 (GBP-155) and 130,000-135,000 (GBP-130), are heat stable, and are soluble. Moreover, antibodies raised against GBP-130 block merozoite invasion in vitro (9).

We have previously isolated cDNA clones that encoded antigenic

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determinants identical with those on the parasite GBP-130 (10). The primary sequence of a cDNA clone revealed that GBP-130 was composed of highly conserved 50 amino acid repeats which were retained in different geographical isolates of P. falciparum.

In the past year the complete structure of the GBP-130 protein was determined from overlapping cDNA clones as well as from genomic DNA (11). The primary structure predicted from these studies for this protein consisted of 774 amino acids. A signal peptide of 69 amino acids was predicted, consistent with the surface localization of this protein on the merozoite, followed by a highly charged region of 155 amino acids, consisting of 35% basic residues and 11 copies of a 50 amino acid repeating sequence. Confirmation of this structure was obtained by expressing this protein and portions of the molecule as a recombinant protein in E. coli and generating both monoclonal and polyclonal antibodies to these proteins. These antisera efficiently blocked the invasion of erythrocytes by merozoites and inhibited the binding of the GBP-130 protein to glycophorin. Immunoelectron microscopy localized the protein to the merozoite surface (12). To assess the role of the 50 amino acid repeats in glycophorin binding, studies were performed in which varying numbers of repeats were expressed as recombinant proteins and their binding to glycophorin was assessed. As the number of repeating units increased in these recombinant proteins, the degree of binding similarly increased, suggesting a role for the unusual repeating structure in this molecule. The GBP-130 protein and gene are conserved in all geographic isolates investigated to date, further suggesting that this protein may play an important role in parasite survival (13).

To assess the potential of this molecule as an effective protective immunogen, in vivo studies are being designed using recombinant protein purified from E. coli as an immunogen for either Aotus or Saimiri monkeys. Discussions with industrial concerns are now in the final stages to obtain the quantities of clinical grade material necessary for these experiments. Collaborations are being formulated with groups capable of performing the monkey studies at this time.

Current studies are focussed on the identification of the amino acid sequences within the 50 amino acid repeat of the GBP 130 protein which are critical to glycophorin binding. To this end, site directed mutagenesis experiments are in progress to alter specific amino acids within the 50 amino acid repeat through the use of synthetic oligonucleotides. Templates have been constructed which encode 1, 2, 3, 6 and 9 50 amino acid repeats into which an origin and packaging site for bacteriophage f1 have been inserted. These plasmid molecules also contain the pBR 322 origin of replication and the tetracycline resistance gene. Upon infection of f$^+$ bacteria harboring these plasmid constructions with a helper phage, tetracycline transducting particles are recovered from the supernatant which are then used to purify single stranded DNA molecules which contain the target sequences of interest. Specific nucleotides will be mutagenized using synthetic oligonucleotides by priming with these molecules and transfecting the resultant double stranded molecules into competent E. coli. Identification of the mutated templates will be pursued by DNA sequence analysis. Re-introduction of the mutated molecules into the expression vector system described for GBP-130 expression (11) followed by glycophorin affinity chromatography as described (11) will assess the relative contribution of amino acid sequences in the GBP-130 molecule in glycophorin binding.

2)   STRUCTURE OF THE KAHRP IN K+ AND K- PARASITES

The significant morbidity and mortality associated with Plasmodium falciparum malaria results, in part, from the sequestration of parasitized erythrocytes in postcapillary venules, which may protect the parasite from splenic clearance (14,15) and contribute to the pathogenesis of cerebral malaria (16). This sequestration has been linked to the expression of parasite-induced knob structures on the surface of the infected erythrocyte which mediate the cytoadherence phenomenon (17,18). While knobs are necessary for cytoadherence, they are not sufficient, requiring both parasite- and host-encoded proteins (19-23). Spontaneous mutants of P. falciparum have been isolated from in vitro cultures which lack the ability to express knobs and fail to cytoadhere (24). A histidine-rich protein has been described which is associated with the knobby phenotype (25) and may be a constituent of the knob (26). Complementary DNA clones for a knob-associated histidine-rich protein (KAHRP) have been isolated and demonstrate that in knobless mutants the gene for this protein has undergone a rearrangement, resulting in a deletion in the 3' coding sequences. Moreover, the chromosome to which the KAHRP maps is rearranged in these mutants, producing a telomeric location of the truncated gene (27). These observations explain the loss of expression of the messenger RNA and protein in such mutants may explain the loss of the knob itself.

In order to distinguish between the possible genetic mechanisms responsible for this chromosomal rearrangement, genomic DNA has been cloned for the KAHRP gene from both K+ and K- parasites for the region spanning the rearrangement in the K- isolates. To complete the analysis of this rearrangement, the DNA corresponding to the chromosome 2 telomeric sequences involved in this rearrangement will be cloned from the K+ isolates, using the rearranged K- KAHRP gene fragment as a probe. Sequence elements which might mediate this rearrangement will be identified by this analysis.

3)   REGULATION OF KAHRP EXPRESSION

Rearrangement of the KAHRP gene in K- isolates results in the loss of accumulation of RNA. The genetic analysis described earlier demonstrated that the 5' end of the gene remained intact, although it had been relocated within 2 kb of a telomere. The mechanism by which RNA accumulation is lost in the K- isolate has been addressed by a detailed study of the transcription of the KAHRP gene in K+ and K- isolates. Nuclei of K+ and K- parasites have been isolated and demonstrated to function in nuclear run-on transcription assays, which measure the RNA polymerase density of a particular sequence. In those experiments, using DNA fragments which correspond to the 5' sequences of the KAHRP gene, preliminary results indicate that transcription of this gene is unaffected by the rearrangement which relocates the gene to a telomeric position. The loss of accumulation of RNA in K- isolates most likely results from the decreased stability of the chimeric mRNA generated as a result of this rearrangement. In order to demonstrate this point, RNA half-life studies are in progress for sequences transcribed from the KAHRP gene in K+ and K- isolates. Similarly, the regulation of RNA accumulation observed for this gene during the intraerythrocytic development of the parasite is being investigated by nuclear run-on transcription and RNA half-life studies. These experiments will address the levels of regulation of expression for this gene in both wild-type and mutant parasites.

The structure of a transcription unit for Plasmodium is a long-range goal of the studies outlined above. Characterization of the 5' end of the mRNA and genomic DNA for the KAHRP gene is in progress to determine if transcription is continuous or discontinuous and the structure of promoter elements in this parasite.

4) ROLE OF THE KAHRP IN KNOB FORMATION AND SEQUESTRATION

The primary structure of the KAHRP has been deduced by nucleotide sequence analysis of the overlapping clones isolated for this protein. An open reading frame of 402 amino acids was identified which encoded a signal sequence, a potential prosequence with an N-linked glycosylation site and a polyhistidine sequence of 60 amino acids (28). These sequence elements are analogous to the HRP of P. lophurae characterized earlier in the laboratory and suggest a functional relationship between these two proteins.

Comparision of these two amino acid sequences, by dot matrix analysis, clearly indicates the polyhistidine tracts of these two proteins. To determine the role of this protein in knob formation and sequestration, recombinant proteins have been expressed in E. coli to various portions of the KAHRP sequence and antisera prepared in rabbits. These antibodies identify a single protein in K+ parasites (FCR-3) of apparent molecular weight of 80-90 kd not expressed in K- isolates. Localization of the protein identified by these antisera by immunofluorescence and immunoelectron-microscopy are currently in progress. Studies are also in progress to define the intracellular route taken by this protein from its site of synthesis to its association with the knob structures on the erythrocyte surface.

The localization of this protein and its orientation relative to the knob structure will provide insight into its possible role in knob formation and sequestration. To address the biological function of this protein, antibodies described above, directed to different portions of the protein are being used to test their effect on cytoadherence in vitro, using the melanoma cell cytoadherence assay. In addition, the possibility that this protein is interacting with other parasite encoded proteins involved in cytoadherence is being studied by using the recombinant proteins as affinity reagents to identify other proteins which are capable of interacting with it.

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